8EHQ-02-15162

SRS
Scientific & Regulatory
SOLUTIONS

8EHQ-0602-15162

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OPPT Document Control Officer
Office of Pollution Prevention and Toxics
U.S. Environmental Protection Agency
1201 Constitution Avenue
Washington, DC 20004

Contain NO CBI

Attn: Mr. Richard Hefter
TSCA § 8(e) Coordinator

Re: TSCA § 8(e) Submission: Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2)

Dear Mr. Hefter:

On behalf of OMG Inc., Scientific & Regulatory Solutions, LLC is submitting the enclosed two mutagenicity studies conducted on the chemical substance described as 'Dyhydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2)' and assigned Chemical Abstract Services Registry Number 68133-90-4. The original studies were conducted in Italy by OMG's German parent company, OMG AG & Co. KG, dmc^2.

OMG's decision to report these studies under TSCA § 8(e) is due to the positive results in both mutagenicity studies. The first study was an *in vitro* reverse mutation study using *Salmonella typhiurium* and *Escherichia coli* performed with and without metabolic activation. The final report, RTC Report No.: 8741-M-04901, indicates that the above referenced substance induced reproducible, large and dose-related increases in the number of revertant colonies, at several dose-levels, with TA98, TA100 and WP2 *uvr*A tester strains, both in the absence and presence of S9 metabolism.

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The second study was an *in vitro* gene mutation study in Chinese hamster V79 cells with and without metabolic activation. OMG AG received a draft report of this study on June 7, 2002. This second study, RTC Study No. 9397, indicates statistically significant large increases in mutant frequency both in the absence and presence of metabolic activation.

OMG manufactures 'dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2)' ('Platinum-EA salt') for commercial distribution in the United States as an abatement catalyst. Manufacturing occurs at the OMG facility located at 3900 South Clinton Avenue, South Plainfield, New Jersey, 07080.

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Production in 2001 was approximately 20 kilograms. OMG does not import this chemical substance and has no plans to increase its 2001 production volume at this time.

OMG is also aware that approximately 700 kg of platinum-EA salt was imported by a subsidiary, International Catalyst Technology, located at 5150 Gilbertsville Highway, Calvert City, Kentucky 42029. The material is produced in Canada by OMG Catalysts Canada Corporation and is used in the production of automobile catalytic converters. The application process converts the platinum-EA salt to the final catalyst, which is dried onto a fixed autocatalyst substrate that becomes part of the finished catalytic converter.

Due to the presence of platinum in the compound, personnel involved in production are required to follow strict industrial hygiene practices. Personal-use respirators and rubberized gloves, and mechanical exhaust are in use during the entire reaction cycle and packaging activities. After careful review, the company has determined that no additional industrial hygiene measures are necessary. The material safety data sheet for this substance has been updated to reflect the potential for mutagenicity based on *in vitro* studies and employees at the OMG and International Catalyst Technology facilities that come in contact with platinum-EA salt have been informed of the results.

If you have any questions, please contact me at 410-480-0955.

Sincerely,

Pamela Kreis

Pamela Kreis
Scientific & Regulatory Solutions

Enclosures

Cc: J. Coney, G. Sommer

RESEARCH TOXICOLOGY CENTRE · ROMA

Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2)
BACTERIAL MUTATION ASSAY
(*S. typhimurium and E. coli*)

FINAL REPORT

RTC Report No.: 8741-M-04901

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Partita IVA 00920611001

RTC Report No.: 8741-M-04901

COMPLIANCE STATEMENT

We, the undersigned, hereby declare that the following report constitutes a true and faithful account of the procedures adopted, and the results obtained in the performance of the study. The aspects of the study conducted by Research Toxicology Centre S.p.A. were performed in accordance with:

A. *"Good Laboratory Practice Regulations"* of the U.S. Food and Drug Administration, Code of Federal Regulations, 21 Part 58, 22 December 1978 and subsequent revisions.

B. Commission Directive 1999/11/EC of 8 March 1999 adapting to technical progress the principles of good laboratory practice as specified in Council Directive 87/18/EEC on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (adoption of the *"OECD principles on Good Laboratory Practice – as revised in 1997"*) and subsequent revisions.

C. Decreto Legislativo 27 gennaio 1992, n. 120 published in the Gazzetta Ufficiale della Repubblica Italiana 18 Febbraio 1992 (adoption of the Commission Directive of 18 December 1989 adapting to technical progress the Annex to Council Directive 88/320/EEC on the inspection and verification of Good Laboratory Practice (90/18/EEC)) and subsequent revisions.

Ornella Scarcella

O. Scarcella, Biol.D.
(Study Director) : Date : 11 Oct 2001

J. Brightwell

J. Brightwell, Ph.D.
(Scientific Director) : Date : 11.10.2001

OMG dmc² division
PC - RP - PS
05-06-02

RTC Report No.: 8741-M-04901

QUALITY ASSURANCE STATEMENT

(Relevant to the aspects of the study conducted by RTC)

Study phases monitored by RTC's QAU according to current relevant Standard Operating Procedures	Quality Assurance Inspections (Day Month Year)		
	Inspection	Report to Study Director	Report to Company Management
PROTOCOL CHECK	05.06.2001	05.06.2001	05.06.2001
PROCESS-BASED INSPECTIONS			
Dose preparation	01.06.2001	-	08.06.2001
Treatment	22.06.2001	-	12.07.2001
Plating out	22.06.2001	-	12.07.2001
Plate scoring	09.07.2001	-	12.07.2001
Other routine inspections of a procedural nature were carried out on activities not directly related to this type of study. The relevant documentation is kept on file although specific inspection dates are not reported here.			
FINAL REPORT Review of this report by RTC's QAU found the reported methods and procedures to describe those used and the results to constitute an accurate representation of the recorded raw data.	Review completed 10/Oct/2001		

M.M. Brunetti, Biol.D.
(Head of Quality Assurance)

10/10/01
Date

OMG dmc² division
PC - RP - PS
05 - 06 - 02

Contents

1. SUMMARY

1.1 The test item Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2) was examined for the ability to induce gene mutations in tester strains of *Salmonella typhimurium* and *Escherichia coli,* as measured by reversion of auxotrophic strains to prototrophy. The five tester strains TA1535, TA1537, TA98, TA100 and WP2 *uvr*A were used. Experiments were performed both in the absence and presence of metabolic activation, using liver S9 fraction from rats pre-treated with phenobarbitone and betanaphthoflavone.
Test item solutions were prepared using sterile distilled water.

1.2 In the toxicity test, the test item was assayed at a maximum dose-level of 5000 µg/plate and four lower dose-levels spaced at approximately half-log intervals: 1580, 500, 158 and 50.0 µg/plate. No signs of toxicity were observed in any tester strain, in the absence or presence of S9 metabolic activation.

1.3 Two main experiments were performed.
In Main Assay I, using the plate incorporation method, the test item was assayed at a maximum dose-level of 5000 µg/plate and at four lower dose-levels separated by two-fold dilutions: 2500, 1250, 625 and 313 µg/plate
Dose-related increases in revertant colonies, which were more than two-fold the control value, were observed in TA98, TA100 and WP2 *uvr*A tester strains, both in the absence and presence of S9 metabolism.

On the basis of these results in Main Assay II, with TA98, TA100 and WP2 *uvr*A the test item was assayed under the same experimental conditions, while with TA1535 and TA1537 tester strains a pre-incubation step was included.

The test item induced reproducible, large and dose-related increases in the number of revertant colonies, which were more than two-fold the control value, with TA98, TA100 and WP2 *uvr*A tester strains in the plate incorporation assays, at the higher dose-levels, both in the absence and presence of S9 metabolism.
Increases in revertant numbers were also observed with TA1537 in the pre-incubation assay. These increases were greater than twice the control value at non toxic dose-levels, both in the absence and presence of S9 metabolism.

1.4 It is concluded that the test item Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2) induces reverse mutation in *Salmonella typhimurium* and *Escherichia coli* under the reported experimental conditions.

2. INTRODUCTION

2.1 Purpose

This report describes experiments performed to assess the mutagenic activity of the test item to *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100, and to *Escherichia coli* strain WP2 *uvr*A using the procedures developed by Ames *et al.*, 1975 and revised by Maron and Ames, 1983.

The study was designed to comply with the experimental methods indicated in:
- EEC Council Directive 2000/32, Annex 4D.
- OECD Guideline for the testing of chemicals No. 471 (Adopted July 1997).
- ICH S2A Genotoxicity: Specific Aspects of Regulatory Tests.

2.2 Principles of the method

Reverse mutation assays employ bacterial strains which are already mutant at a locus whose phenotypic effects are easily detected. The *Salmonella* tester strains have mutations causing dependence on a particular amino acid (histidine) for growth. The ability of test items to cause reverse mutations (reversions) to histidine-independence can easily be measured. The *E. coli* tester strains of the WP2 series are similarly mutant at the tryptophan locus.

Since many chemicals only demonstrate mutagenic activity after metabolism to reactive forms, in order to detect these "indirect mutagens" the test is performed in the presence and absence of a rat liver metabolising system.

2.3 Study organisation

Sponsor

OMG AG & Co. KG
dmc²
Precious Metals Chemistry
Rodenbacher Chaussee, 4
D-63403 Hanau - Wolfgang
Gemany

Location of Study:

Research Toxicology Centre S.p.A.
Genetic Toxicology Department
Via Tito Speri, 12
00040 Pomezia (Roma)
Italy

Principal dates:

Study protocol approved by Study Director: 23-Apr-2001
Study commenced: 01-June-2001 (Toxicity assay treatment)
Study completed: 25-June-2001 (Completion of scoring Main Assay II)

Study Monitor:

Karl – Heinz Krebs

Study Director:

O. Scarcella, Biol. D.

Archiving:

The original data arising from this study and a copy of the final report consigned will be stored in the archives of Research Toxicology Centre S.p.A. for a period of at least five years from the date of consignment of the report. At the completion of this period the Sponsor will be contacted for despatch or disposal of the material, or further archiving. An aliquot of the test item will be retained within the archives of the testing facility for a period of ten years after which it will be destroyed.

3. MATERIALS AND METHODS

3.1 Test item

A 100 g sample of Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2), labelled as Bis Ethanolamoniumhydoxoplatinat Lsg (Batch No.: A-198-01), was received from DEGUSSA DMC-2 on 18-May-2001 and was stored within the Formulation Unit, at RTC, at +4°C in desiccator. The test item, an orange liquid, was contained in an opaque plastic bottle. On 28-May-2001 a 1.5 g sub-sample of the test item was transferred from the Formulation Unit to the Department of Genetic and Cellular Toxicology and stored under the same conditions.

Solutions of the test item, as received, were prepared immediately before use in sterile distilled water and filter sterilized through a 0.45 µm filter. Solutions were prepared on a weight/volume basis without correction for the displacement due to the volume of the test item. Concentrations were expressed in terms of material as received. No assay of test item stability, nor its concentration and homogeneity in solvent were undertaken. All dose-levels in this report are expressed to three significant figures.

3.2 Control items

The solvent used in this study was sterile distilled water (Bieffe Medital S.p.A. batch 00L18 05).

Positive control treatments used solutions prepared as follows:

Sodium azide (Fluka AG, batch 221999 1081) in distilled water.
9-Aminoacridine (ICN K&K Laboratories, batch 12058-A) in DMSO.
2-Nitrofluorene (EGA Chemie, batch 12532) in DMSO.
2-Aminoanthracene (Sigma, batch 58F-3462) in DMSO.
Methylmethanesulphonate (MMS) (Fluka AG, batch 359316/153696) in distilled water.
Dimethylsulphoxide (DMSO, Fluka AG, batch 401105/1 21100).

3.3 Media

The following growth media were used:

Nutrient Broth: Oxoid Nutrient Broth No 2 was prepared at a concentration of 2.5% in distilled water and autoclaved prior to use.
This was used for the preparation of liquid cultures of the tester strains.

Nutrient Agar: Oxoid Nutrient Broth No 2 (25g) and Difco Bacto-agar (15g) were added to distilled water (1 litre) and autoclaved.
The solutions were then poured into 9 cm plastic Petri dishes and allowed to solidify and dry before use. These plates were used for the non-selective growth of the tester strains.

Minimal Agar: Minimal medium agar was prepared as 1.5% Difco Bacto-agar in Vogel-Bonner Medium E, with 2% Glucose, and poured into 9 cm plastic Petri dishes.

Top Agar: "Top Agar" (overlay agar) was prepared as 0.6% Difco Bacto-agar + 0.5% NaCl in distilled water. Prior to use 10 ml of a sterile solution of 0.5 mM Biotin + 0.5 mM Histidine (or 0.5 mM tryptophan) was added to the top agar (100 ml).

3.4 S9 tissue homogenate

Two batches of S9 tissue homogenate (designated 01/6 and 01/9) were used in this study and had the following characteristics:

S9 Batch	Protein content (mg/ml)	Aminopyrine demethylase activity (µM/g liver/5 min, formaldehyde production)
01/6	37.1 ± 2.41	3.89 ± 0.26
01/9	37.4 ± 0.65	3.64 ± 0.27

The S9 tissue fractions were prepared from the livers of five young male Sprague-Dawley rats which had received prior treatment with phenobarbital and betanaphthoflavone to induce high levels of xenobiotic metabolising enzymes. The efficacy of the S9 tissue fractions was previously checked in an Ames test and produced acceptable responses with the indirect mutagens 2-aminoanthracene and benzo(a)pyrene, using *S. typhimurium* tester strain TA100.

The mixture of S9 tissue fraction and cofactors (S9 mix) was prepared as follows (for each 10 ml):

S9 tissue fraction	1.0 ml
NADP (100 mM)	0.4 ml
G-6-P (100 mM)	0.5 ml
KCl (330 mM)	1.0 ml
MgCl2 (100 mM)	0.8 ml
Phosphate buffer (pH 7.4, 200 mM)	5.0 ml
Distilled Water	1.3 ml
	10.0 ml

3.5 Bacterial strains

Four strains of *Salmonella typhimurium* (TA1535, TA1537, TA98 and TA100) and a strain of *Escherichia coli* (WP2 *uvr*A) were used in this study. Permanent stocks of these strains are kept at -80°C in RTC. Overnight subcultures of these stocks were prepared for each day's work.
Bacteria were taken from vials of frozen cultures, which had been checked for the presence of the appropriate genetic markers, as follows:

Histidine requirement	:	No Growth on Minimal plates + Biotin. Growth on Minimal plates + Biotin + Histidine.
Tryptophan requirement	:	No Growth on Minimal agar plates Growth on Minimal plates + Tryptophan.
*uvr*A, *uvr*B	:	Sensitivity to UV irradiation.
rfa	:	Sensitivity to Crystal Violet.
pKM101	:	Resistance to Ampicillin.

Bacterial cultures in liquid and on agar were clearly identified with their identity.

3.6 Methods

3.6.1 Preliminary toxicity test

A preliminary toxicity test was undertaken in order to select the concentrations of the test item to be used in the main assays. In this test a wide range of dose-levels of the test item, set at half-log intervals, was used. Treatments were performed both in the absence and presence of S9 metabolism using the plate incorporation method; a single plate was used at each test point and positive controls were not included.

3.6.2 Main experiments

Two experiments were performed including negative and positive controls in the absence and presence of an S9 metabolising system. The results presented for TA100 in Main Assay I were obtained in a repeated experiment. An additional experiment with this tester strain was performed, since unacceptably high negative control values were obtained. Data obtained in that experiment are not presented, but are retained in the study file and archived as indicated in the Study Protocol.

Three replicate plates were used at each test point.

In addition, plates were prepared to check the sterility of the test item solutions and the S9 mix, and dilutions of the bacterial cultures were plated on nutrient agar plates to establish the number of bacteria in the cultures.

Using a plate-incorporation method, the components of the assay (the tester strain bacteria, the test item and S9 mix or phosphate buffer) were added to molten overlay agar and vortexed. The mixture was then poured onto the surface of a minimal medium agar plate, and allowed to solidify prior to incubation.

The overlay mixture was composed as follows:

(i)	Overlay agar (held at 45°C)	2 ml
(ii)	Test or control item solution	0.1 ml
(iii)	S9 mix or phosphate buffer (pH 7.4, 0.1 M)	0.5 ml
(iv)	Bacterial suspension	0.1 ml

In the pre-incubation method the components were added in turn to an empty test-tube:

(i)	Bacterial suspension	0.1 ml
(ii)	Test item solution	0.1 ml
	(control item solution)	(0.05) ml
(iii)	S9 mix or phosphate buffer (pH 7.4, 0.1 M)	0.5 ml

The incubate was vortexed and placed at 37°C for 30 minutes. Two ml of overlay agar was then added and the mixture vortexed again and poured onto the surface of a minimal medium agar plate and allowed to solidify.

3.6.3 Incubation and scoring

The prepared plates were inverted and incubated for approximately 72 hours at 37°C. After this period of incubation, the scoring was effected by counting the number of revertant colonies on each plate.

4. RESULTS

4.1 Solubility test

The test item was found to be soluble in sterile distilled water at a concentration of 50 mg/ml. Since 100 µl of the test item solution are used in the preparation of each plate, this permitted a maximum concentration of 5000 µg/plate to be used in the toxicity test.

4.2 Toxicity test

The test item was assayed at a maximum dose-level of 5000 µg/plate and at four lower dose-levels spaced at approximately half-log intervals: 1580, 500, 158 and 50.0 µg/plate. Results are presented in Tables 1 and 2.
No signs of toxicity were observed in any tester strain, in the absence or presence of S9 metabolic activation.

4.3 Assay for reverse mutation

Two experiments were performed; individual plate counts for these tests, and the mean and standard error of the mean for each test point, together with statistical analysis are presented in Tables 3 to 12.

In Main Assay I, using the plate incorporation method, the test item was assayed at a maximum dose-level of 5000 µg/plate and at four lower dose-levels separated by two-fold dilutions: 2500, 1250, 625 and 313 µg/plate
Dose-related increases in revertant colonies, which were greater than twice the control values, were observed in TA98, TA100 and WP2 *uvr*A tester strains, both in the absence and presence of S9 metabolism. These increases were more pronounced in WP2 *uvr*A.

On the basis of these results, in Main Assay II the test item was assayed under the same experimental conditions with TA98, TA100 and WP2 *uvr*A. Treatments of TA1535 and TA1537 tester strains included a pre-incubation step and used the same dose-levels employed in Main Assay I.

The test item induced reproducible, large and dose-related increases in the number of revertant colonies, which were more than two-fold the control value, with TA98, TA100 and WP2 *uvr*A tester strains, at higher dose-levels, both in the absence and presence of S9 metabolism.
Increases in revertant numbers were also observed with TA1537 in the pre-incubation assay. These increases were greater than twice the control values at non toxic dose-levels, both in the absence and presence of S9 metabolism.

The sterility of the S9 mix and the test item solutions was confirmed by the absence of colonies on additional agar plates spread separately with these solutions. Marked increases in revertant numbers were obtained in these tests following treatment with the positive control items, indicating that the assay system was functioning correctly.

5. ANALYSIS OF RESULTS

5.1 Criteria for outcome of the assays

For the test item to be considered mutagenic, two-fold (or more) increases in mean revertant numbers must be observed at two consecutive dose-levels or at the highest practicable dose-level only. In addition, there must be evidence of a dose-response relationship showing increasing numbers of mutant colonies with increasing dose-levels.

5.2 Evaluation

The test item induced reproducible, large and dose-related increases in the number of revertant colonies, at several dose-levels, with TA98, TA100 and WP2 *uvr*A tester strains, both in the absence and presence of S9 metabolism. Increases in revertant numbers which were greater than twice the control values, were also observed with TA1537 following the pre-incubation step, at non toxic dose-levels, both in the absence and presence of S9 metabolism.
On the basis of the stated criteria it must be concluded that the test item Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2) is mutagenic to *S. typhimurium* and *E. coli* under the reported experimental conditions.

6. CONCLUSION

It is concluded that the test item Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2) induces reverse mutation in *Salmonella typhimurium* and *Escherichia coli* under the reported experimental conditions.

7. KEY TO TABLES 1-12

7.1 Structure of Tables 3-12

These tables show, for each *Salmonella typhimurium* or *Escherichia coli* tester strain, the individual plate counts obtained for the negative and positive controls, and at each dose-level of the test item. The mean number of revertant colonies and standard error of the mean are also presented. The "untreated" plates receive no treatment. The titre of the bacterial cultures is given (million cells/plate).

7.2 Regression line

i) The regression analysis fits a regression line to the data by the least squares method, after square root transformation of the plate counts to satisfy normal distribution and homoscedasticity assumptions. The regression equation is expressed as:

$y = a + bx$

where y = transformed revertant numbers
a = intercept
b = slope value
x = dose-level (in the units given).

ii) The regression line includes the untreated control data.

iii) Regression lines are calculated using a minimum of the three lowest dose-levels, and then including the further dose-levels in turn. The correlation co-efficient (r), the value of students "t" statistic, and the p-value for the regression lines are also given.

7.3 Abbreviations

C : Contaminated plates
\- : Not calculated

8. TABLES 1 TO 12

Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2):

BACTERIAL MUTATION ASSAY (*S. typhimurium and E.coli*)

TABLE 1 - WITHOUT METABOLIC ACTIVATION

STUDY NO.: 8741

SOLVENT: sterile distilled water

EXPERIMENT: Toxicity test

Dose-level (µg/plate)	TA-1535 Rev/pl.	TA-1537 Rev/pl.	TA-98 Rev/pl.	TA-100 Rev/pl.	WP2 uvrA Rev/pl.
Untreated	16	18	31	141	31
50.0	25	14	32	199	52
158	22	20	54	210	82
500	14	15	64	282	163
1580	19	17	52	267	311
5000	13	10	87	381	595

Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2):

BACTERIAL MUTATION ASSAY (*S. typhimurium and E.coli*)

TABLE 2 - WITH METABOLIC ACTIVATION

STUDY NO.: 8741

SOLVENT: sterile distilled water

EXPERIMENT: Toxicity test

Dose-level (µg/plate)	TA-1535 Rev/pl.	TA-1537 Rev/pl.	TA-98 Rev/pl.	TA-100 Rev/pl.	WP2 uvrA Rev/pl.
Untreated	21	20	36	163	34
50.0	13	24	43	258	48
158	11	21	45	325	94
500	19	23	66	391	193
1580	12	20	117	517	443
5000	11	19	247	674	182

Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2):

BACTERIAL MUTATION ASSAY (*S. typhimurium and E.coli*)

TABLE 3 - Experiment I - Plate incorporation method -

STUDY NO.: 8741

SOLVENT: sterile distilled water

Strain: TA1535 Titre: 210

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	17	11	16	15	1.9	13	18	13	15	1.7
313	19	18	13	17	1.9	15	17	15	16	0.7
625	10	12	16	13	1.8	13	14	16	14	0.9
1250	12	8	14	11	1.8	13	16	14	14	0.9
2500	10	9	13	11	1.2	10	15	10	12	1.7
5000	9	11	7	9	1.2	10	12	10	11	0.7

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	3.944	-0.0004	-0.27224	0.7485	0.47852
1 - 4	-	3.955	-0.0005	-0.49592	1.8060	0.10107
1 - 5	-	3.867	-0.0003	-0.54734	2.3580	0.03470
1 - 6	-	3.793	-0.0002	-0.63734	3.3083	0.00444
1 - 3	+	3.870	-0.0001	-0.06502	0.1724	0.86801
1 - 4	+	3.873	-0.0001	-0.15413	0.4933	0.63246
1 - 5	+	3.925	-0.0002	-0.56361	2.4601	0.02866
1 - 6	+	3.885	-0.0001	-0.69859	3.9053	0.00126

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	17	11	16	15	1.9
Sodium Azide	1 µg/pl	-	658	657	642	652	5.2
DMSO	100 µl/pl	+	12	20	12	15	2.7
2-Aminoanthracene	1 µg/pl	+	183	159	C	171	12.0

Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2):

BACTERIAL MUTATION ASSAY (*S. typhimurium and E.coli*)

TABLE 4 - Experiment I - Plate incorporation method -

STUDY NO.: 8741

SOLVENT: sterile distilled water

Strain: TA1537 Titre: 209

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	13	10	16	13	1.7	21	19	15	18	1.8
313	18	19	19	19	0.3	18	15	18	17	1.0
625	19	19	18	19	0.3	15	18	15	16	1.0
1250	14	17	12	14	1.5	13	17	15	15	1.2
2500	12	15	16	14	1.2	12	9	12	11	1.0
5000	13	15	13	14	0.7	10	14	11	12	1.2

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	3.711	0.0012	0.74696	2.9724	0.02074
1 - 4	-	3.990	0.0000	0.02399	0.0759	0.94101
1 - 5	-	4.036	-0.0001	-0.19798	0.7283	0.47937
1 - 6	-	4.025	-0.0001	-0.32717	1.3849	0.18509
1 - 3	+	4.267	-0.0004	-0.45076	1.3360	0.22334
1 - 4	+	4.237	-0.0003	-0.54973	2.0810	0.06409
1 - 5	+	4.260	-0.0004	-0.82695	5.3028	0.00014
1 - 6	+	4.117	-0.0002	-0.73107	4.2859	0.00057

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
DMSO	100 µl/pl	-	19	17	16	17	0.9
9-Aminoacridine	50 µg/pl	-	106	91	103	100	4.6
DMSO	100 µl/pl	+	19	18	21	19	0.9
2-Aminoanthracene	1 µg/pl	+	89	90	81	87	2.8

Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2):

BACTERIAL MUTATION ASSAY (*S. typhimurium and E.coli*)

TABLE 5 - Experiment I - Plate incorporation method -

STUDY NO.: 8741

SOLVENT: sterile distilled water

Strain: WP2 *uvr*A Titre: 240

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	30	31	22	28	2.8	29	34	33	32	1.5
313	160	138	127	142	9.7	148	152	168	156	6.1
625	183	195	185	188	3.7	240	233	240	238	2.3
1250	244	224	240	236	6.1	291	319	305	305	8.1
2500	326	335	330	330	2.6	360	407	408	392	15.8
5000	435	431	460	442	9.1	437	436	422	432	4.8

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	6.047	0.0135	0.94370	7.5478	0.00013
1 - 4	-	7.568	0.0073	0.87111	5.6093	0.00022
1 - 5	-	8.896	0.0042	0.86139	6.1145	0.00004
1 - 6	-	10.194	0.0025	0.85748	6.6664	0.00001
1 - 3	+	6.300	0.0156	0.97300	11.1537	0.00001
1 - 4	+	7.975	0.0087	0.90280	6.6384	0.00006
1 - 5	+	9.732	0.0047	0.85252	5.8806	0.00005
1 - 6	+	11.528	0.0023	0.77729	4.9419	0.00015

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	30	31	22	28	2.8
MMS	500 µg/pl	-	205	196	204	202	2.8
DMSO	100 µl/pl	+	23	30	29	27	2.2
2-Aminoanthracene	10 µg/pl	+	219	187	192	199	9.9

Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2):

BACTERIAL MUTATION ASSAY (*S. typhimurium and E.coli*)

TABLE 6 - Experiment I - Plate incorporation method -

STUDY NO.: 8741

SOLVENT: sterile distilled water

Strain: TA98 Titre: 217

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	32	32	43	36	3.7	49	50	48	49	0.6
313	49	52	41	47	3.3	71	65	72	69	2.2
625	54	48	56	53	2.4	87	79	71	79	4.6
1250	57	61	53	57	2.3	88	93	95	92	2.1
2500	74	62	86	74	6.9	160	152	160	157	2.7
5000	120	140	145	135	7.6	210	217	218	215	2.5

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	6.045	0.0021	0.82312	3.8350	0.00642
1 - 4	-	6.264	0.0012	0.80292	4.2595	0.00166
1 - 5	-	6.372	0.0009	0.87339	6.4660	0.00002
1 - 6	-	6.287	0.0010	0.96698	15.1760	0.00000
1 - 3	+	7.127	0.0030	0.93111	6.7543	0.00026
1 - 4	+	7.387	0.0019	0.91991	7.4184	0.00002
1 - 5	+	7.334	0.0021	0.98174	18.6079	0.00000
1 - 6	+	7.760	0.0015	0.97217	16.5998	0.00000

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
DMSO	100 µl/pl	-	36	31	21	29	4.4
2-Nitrofluorene	2 µg/pl	-	263	237	225	242	11.2
DMSO	100 µl/pl	+	50	46	49	48	1.2
2-Aminoanthracene	1 µg/pl	+	675	670	696	680	8.0

Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2):

BACTERIAL MUTATION ASSAY (*S. typhimurium and E.coli*)

TABLE 7 - Experiment I - Plate incorporation method -

STUDY NO.: 8741

SOLVENT: sterile distilled water

Strain: TA100 Titre: 213

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	154	148	148	150	2.0	135	139	134	136	1.5
313	168	162	171	167	2.6	244	236	240	240	2.3
625	188	186	195	190	2.7	351	329	362	347	9.7
1250	207	198	192	199	4.4	385	358	340	361	13.1
2500	261	287	245	264	12.2	500	461	478	480	11.3
5000	371	360	382	371	6.4	559	545	589	564	13.0

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	12.218	0.0024	0.97555	11.7438	0.00001
1 - 4	-	12.450	0.0015	0.92068	7.4589	0.00002
1 - 5	-	12.434	0.0015	0.96730	13.7497	0.00000
1 - 6	-	12.544	0.0014	0.98839	26.0226	0.00000
1 - 3	+	11.774	0.0112	0.99525	27.0415	0.00000
1 - 4	+	13.120	0.0056	0.87551	5.7299	0.00019
1 - 5	+	14.044	0.0035	0.88573	6.8796	0.00001
1 - 6	+	15.165	0.0020	0.86142	6.7843	0.00000

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	148	148	154	150	2.0
Sodium Azide	1 µg/pl	-	951	1062	984	999	32.9
DMSO	100 µl/pl	+	136	134	141	137	2.1
2-Aminoanthracene	1 µg/pl	+	1172	1216	1221	1203	15.6

Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2):

BACTERIAL MUTATION ASSAY (*S. typhimurium and E.coli*)

TABLE 8 - Experiment II - Plate incorporation method -

STUDY NO.: 8741

SOLVENT: sterile distilled water

Strain: WP2 *uvrA* Titre: 253

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	30	29	27	29	0.9	40	33	37	37	2.0
313	87	71	95	84	7.1	97	114	116	109	6.0
625	149	138	136	141	4.0	164	155	149	156	4.4
1250	174	198	171	181	8.5	224	204	232	220	8.3
2500	273	257	287	272	8.7	279	303	291	291	6.9
5000	351	327	333	337	7.2	325	283	297	302	12.3

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	5.536	0.0104	0.98673	16.0779	0.00000
1 - 4	-	6.562	0.0062	0.92835	7.8976	0.00001
1 - 5	-	7.541	0.0040	0.92192	8.5809	0.00000
1 - 6	-	8.835	0.0022	0.87876	7.3650	0.00000
1 - 3	+	6.436	0.0103	0.97264	11.0763	0.00001
1 - 4	+	7.334	0.0066	0.94139	8.8255	0.00000
1 - 5	+	8.525	0.0039	0.90550	7.6939	0.00000
1 - 6	+	10.040	0.0019	0.80096	5.3512	0.00006

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	30	29	27	29	0.9
MMS	500 µg/pl	-	169	178	159	169	5.5
DMSO	100 µl/pl	+	38	31	30	33	2.5
2-Aminoanthracene	10 µg/pl	+	192	198	207	199	4.4

Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2):

BACTERIAL MUTATION ASSAY (*S. typhimurium and E.coli*)

TABLE 9 - Experiment II - Plate incorporation method -

STUDY NO.: 8741

SOLVENT: sterile distilled water

Strain: TA98 Titre: 228

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	31	37	34	34	1.7	42	37	39	39	1.5
313	41	54	42	46	4.2	85	101	94	93	4.6
625	55	54	51	53	1.2	123	91	82	99	12.4
1250	65	76	81	74	4.7	111	131	117	120	5.9
2500	81	78	92	84	4.3	185	177	150	171	10.6
5000	109	116	105	110	3.2	220	218	235	224	5.4

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	5.887	0.0024	0.89749	5.3841	0.00103
1 - 4	-	5.935	0.0022	0.95036	9.6590	0.00000
1 - 5	-	6.319	0.0013	0.90752	7.7907	0.00000
1 - 6	-	6.640	0.0009	0.91923	9.3390	0.00000
1 - 3	+	6.792	0.0058	0.84944	4.2591	0.00375
1 - 4	+	7.415	0.0032	0.82081	4.5441	0.00107
1 - 5	+	7.847	0.0023	0.88058	6.6996	0.00001
1 - 6	+	8.439	0.0015	0.89860	8.1918	0.00000

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
DMSO	100 µl/pl	-	34	28	34	32	2.0
2-Nitrofluorene	2 µg/pl	-	199	206	187	197	5.5
DMSO	100 µl/pl	+	39	42	45	42	1.7
2-Aminoanthracene	1 µg/pl	+	632	635	625	631	3.0

Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2):

BACTERIAL MUTATION ASSAY (*S. typhimurium and E.coli*)

TABLE 10 - Experiment II - Plate incorporation method -

STUDY NO.: 8741

SOLVENT: sterile distilled water

Strain: TA100 Titre: 215

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	131	125	133	130	2.4	113	129	120	121	4.6
313	170	163	188	174	7.4	241	241	272	251	10.3
625	219	192	214	208	8.3	293	297	311	300	5.5
1250	284	240	253	259	13.1	333	363	350	349	8.7
2500	289	300	271	287	8.5	446	417	417	427	9.7
5000	415	398	372	395	12.5	507	503	494	501	3.8

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	11.474	0.0049	0.95996	9.0661	0.00004
1 - 4	-	11.769	0.0037	0.95535	10.2246	0.00000
1 - 5	-	12.465	0.0021	0.89820	7.3672	0.00001
1 - 6	-	12.881	0.0015	0.93510	10.5548	0.00000
1 - 3	+	11.542	0.0102	0.94860	7.9307	0.00010
1 - 4	+	12.657	0.0056	0.88073	5.8804	0.00016
1 - 5	+	13.697	0.0032	0.86085	6.0997	0.00004
1 - 6	+	14.740	0.0018	0.84030	6.2000	0.00001

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	131	125	133	130	2.4
Sodium Azide	1 µg/pl	-	900	910	970	927	21.9
DMSO	100 µl/pl	+	127	131	129	129	1.2
2-Aminoanthracene	1 µg/pl	+	1070	1114	1124	1103	16.6

Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2):

BACTERIAL MUTATION ASSAY (*S. typhimurium and E.coli*)

TABLE 11 - Experiment II - Preincubation method -

STUDY NO.: 8741

SOLVENT: sterile distilled water

Strain: TA1535 Titre: 222

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	16	18	21	18	1.5	12	15	15	14	1.0
313	13	17	13	14	1.3	12	11	13	12	0.6
625	12	13	12	12	0.3	13	15	12	13	0.9
1250	12	12	16	13	1.3	13	13	16	14	1.0
2500	10	10	7	9	1.0	10	7	10	9	1.0
5000	14	4	5	8	3.2	4	7	7	6	1.0

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	4.237	-0.0012	-0.83194	3.9669	0.00541
1 - 4	-	4.050	-0.0005	-0.58234	2.2652	0.04695
1 - 5	-	4.037	-0.0004	-0.79694	4.7569	0.00037
1 - 6	-	3.931	-0.0003	-0.74847	4.5146	0.00035
1 - 3	+	3.660	-0.0001	-0.18318	0.4930	0.63710
1 - 4	+	3.609	0.0001	0.15340	0.4909	0.63408
1 - 5	+	3.747	-0.0002	-0.64922	3.0776	0.00882
1 - 6	+	3.758	-0.0003	-0.86167	6.7920	0.00000

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	16	18	21	18	1.5
Sodium Azide	1 µg/pl	-	651	610	633	631	11.9
DMSO	50 µl/pl	+	11	17	13	14	1.8
2-Aminoanthracene	1 µg/pl	+	95	92	90	92	1.5

Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2):

BACTERIAL MUTATION ASSAY (*S. typhimurium and E.coli*)

TABLE 12 - Experiment II - Preincubation method -

STUDY NO.: 8741

SOLVENT: sterile distilled water

Strain: TA1537 Titre: 204

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	12	14	16	14	1.2	19	22	20	20	0.9
313	63	61	73	66	3.7	51	41	60	51	5.5
625	82	64	61	69	6.6	91	100	85	92	4.4
1250	38	32	48	39	4.7	94	90	83	89	3.2
2500	13	19	14	15	1.9	50	52	72	58	7.0
5000	11	13	16	13	1.5	22	23	26	24	1.2

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	4.428	0.0073	0.86979	4.6639	0.00230
1 - 4	-	5.887	0.0013	0.31708	1.0572	0.31527
1 - 5	-	6.799	-0.0008	-0.35055	1.3496	0.20019
1 - 6	-	6.683	-0.0006	-0.53678	2.5448	0.02163
1 - 3	+	4.522	0.0081	0.98394	14.5845	0.00000
1 - 4	+	5.573	0.0038	0.83891	4.8742	0.00065
1 - 5	+	6.886	0.0008	0.37554	1.4610	0.16777
1 - 6	+	7.751	-0.0004	-0.30137	1.2643	0.22424

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
DMSO	50 µl/pl	-	17	22	17	19	1.7
9-Aminoacridine	50 µg/pl	-	120	167	114	134	16.8
DMSO	50 µl/pl	+	29	28	21	26	2.5
2-Aminoanthracene	1 µg/pl	+	86	97	96	93	3.5

9. APPENDIX I - Historical Control Data

WITHOUT METABOLIC ACTIVATION

	Untreated *Plate incorporation*	**Untreated** *Pre-incubation*	**Positive control** *Plate incorporation*	**Positive control** *Pre-incubation*
TA1535				
Mean value	19	19	520	516
SD	2.8	2.7	74.5	83.8
n	222	105	222	105
TA1537				
Mean value	17	18	148	123
SD	2.3	1.8	50.3	37.8
n	227	105	227	105
TA98				
Mean value	31	30	224	211
SD	3.1	2.3	31.2	27.0
n	227	102	227	102
TA100				
Mean value	152	135	720	739
SD	18.8	13.7	112.2	128.6
n	228	104	228	104
WP2 *uvrA*				
Mean value	29	30	159	191
SD	5.2	7.0	49.6	111.5
n	6	8	6	8

SD : standard deviation
n : number of experiments

WITH METABOLIC ACTIVATION

	Untreated *Plate incorporation*	**Untreated** *Pre-incubation*	**Positive control** *Plate incorporation*	**Positive control** *Pre-incubation*
TA1535				
Mean value	17	16	154	96
SD	2.4	2.0	32.0	15.5
n	220	105	220	105
TA1537				
Mean value	22	23	120	87
SD	2.8	2.0	25.1	13.6
n	224	103	224	103
TA98				
Mean value	44	42	1079	1008
SD	5.4	4.7	226.2	194.3
n	232	98	232	98
TA100				
Mean value	166	150	1276	1144
SD	18.7	14.9	265.0	181.6
n	235	99	235	99
WP2 *uvrA*				
Mean value	36	37	295	284
SD	8.9	9.4	75.4	91.9
n	6	8	6	8

SD : standard deviation
n : number of experiments

10. APPENDIX II - Study Protocol

Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2)
GENE MUTATION IN
CHINESE HAMSTER V79 CELLS

DRAFT REPORT

RTC Study no.: 9397

Sponsor:
OMG AG & Co. KG
dmc²
Precious Metals Chemistry
Rodenbacher Chaussee, 4
D-63403 Hanau - Wolfgang
Gemany

COMPLIANCE STATEMENT

We, the undersigned, hereby declare that the following report constitutes a true and faithful account of the procedures adopted and the results obtained, in the performance of this study. The aspects of the study conducted by Research Toxicology Centre S.p.A. were performed in accordance with:

A. *"Good Laboratory Practice Regulations"* of the U.S. Food and Drug Administration, Code of Federal Regulations, 21 Part 58, 22 December 1978 and subsequent revisions.

B. Commission Directive 1999/11/EC of 8 March 1999 adapting to technical progress the principles of good laboratory practice as specified in Council Directive 87/18/EEC on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical items (adoption of the *"OECD principles on Good Laboratory Practice – as revised in 1997"*) and subsequent revisions.

C. Decreto Legislativo 27 Gennaio 1992, n. 120 published in the Gazzetta Ufficiale della Repubblica Italiana 18 Febbraio 1992 (adoption of the Commission Directive of 18 December 1989 adapting to technical progress the Annex to Council Directive 88/320/EEC on the inspection and verification of Good Laboratory Practice (90/18/EEC)) and subsequent revisions.

S. Cinelli, Biol.D.
(Study Director) : Date :

J. Brightwell, Ph.D.
(Scientific Director): Date :

QUALITY ASSURANCE STATEMENT

(Relevant to the aspects of the study conducted by RTC)

Study phases monitored by RTC's QAU according to current relevant Standard Operating Procedures	Quality Assurance Inspections (Day Month Year)		
	Inspection	Report to Study Director	Report to Company Management
PROTOCOL CHECK			
PROCESS-BASED INSPECTIONS			
Other routine inspections of a procedural nature were carried out on activities not directly related to this type of study. The relevant documentation is kept on file although specific inspection dates are not reported here.			
FINAL REPORT Review of this report by RTC's QAU found the reported methods and procedures to describe those used and the results to constitute an accurate representation of the recorded raw data.	Review completed		

M.M. Brunetti, Biol.D.
(Head of Quality Assurance)

Date

Contents

1. SUMMARY

1.1 The test item Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2) was examined for mutagenic activity by assaying for the induction of 6-thioguanine resistant mutants in Chinese hamster V79 cells after *in vitro* treatment. Experiments were performed both in the absence and presence of metabolic activation, using liver S9 fraction from rats pre-treated with phenobarbitone and betanaphthoflavone.
Test item solutions were prepared using culture medium.

1.2 A preliminary cytotoxicity assay was performed. The test item was assayed at a maximum dose-level of 5000 µg/ml and a wide range of lower dose-levels: 2500, 1250, 625, 313, 156, 78.1, 39.1 and 19.5 µg/ml. In the absence of S9 metabolic activation, toxicity after treatment with the five higher dose-levels was severe and survival was reduced to below or near the limit of detection. At the remaining concentrations, dose-related toxicity was observed reducing survival to 5% of the concurrent control value at 156 µg/ml. In the presence of S9 metabolism, survival was reduced to 40% of the negative control value at the highest dose-level. On the basis of these results, the maximum dose-levels for the mutation assay were selected as 78.1 µg/ml for treatment in the absence of S9 metabolism and 5000 µg/ml in the presence.

1.3 A main assay for mutation to 6-thioguanine resistance was performed using dose-levels indicated in the following table:

Assay No.:	S9	Dose-level (µg/ml)
1	-	78.1, 39.1, 19.5, 9.77, 4.88 and 2.44
1	+	5000, 2500, 1250, 625 and 313

Dose-related and large increases in mutant numbers or mutant frequency were observed following treatment with the test item, in the absence and presence of S9 metabolism. These increases were greater than five-fold the spontaneous mutation frequency, in both replicate cultures when analysed separately, at both expression times and so can be considered as clear evidence of mutation induction.

Negative and positive control treatments were included in each mutation experiment in the absence and presence of S9 metabolism. Marked increases were obtained with the positive control treatments indicating the correct functioning of the assay system.

1.4 It is concluded that Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2) induces gene mutation in Chinese hamster V79 cells after *in vitro* treatment in the absence and presence of S9 metabolic activation, under the reported experimental conditions.

2. INTRODUCTION

2.1 Purpose

This report describes experiments performed to assess the mutagenic activity of the test item by assaying for the induction of 6-thioguanine resistant mutants in Chinese hamster V79 cells after *in vitro* treatment (in the absence and presence of S9 metabolic activation).

The study was designed to comply with the experimental methods indicated in:

- EEC Council Directive 2000/32, Annex 4E.
- OECD Guideline for the testing of chemicals No. 476 (Adopted July 1997).

2.2 Principles of the method

The gene mutation assay method used here is based on the identification of V79 fibroblast colonies which have become resistant to a toxic purine analogue (6-thioguanine). This analogue can be metabolised by the enzyme hypoxanthine-guaninphosphoribosyl-transferase (HGPRT) into nucleotides, which are used in nucleic acid synthesis resulting in the death of HGPRT-competent cells. HGPRT-deficient cells, which are presumed to arise through mutations in the HGPRT gene, cannot metabolise 6-thioguanine and thus survive and grow in its presence.
The mutations induced are recessive. However, since the gene which codes for the HGPRT enzyme is located on the X chromosome, of which only one copy is present in male cells, a single mutation is sufficient for the mutant genotype to be observed. The cells used, Chinese hamster V79 cells, are derived from a culture of embryonic lung tissue of male Chinese hamster (*Cricetulus griseus*). The use of the HGPRT mutation system in Chinese hamster V79 cells has been well characterised and validated (Bradley *et al.* 1981) and is accepted by many regulatory authorities.

2.3 Study organisation

Sponsor

OMG AG & Co. KG
dmc²
Precious Metals Chemistry
Rodenbacher Chaussee, 4
D-63403 Hanau - Wolfgang
Gemany

Study Monitor:

Karl-Heinz Krebs

Location of Study

Genetic and Cellular Toxicology Department
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Principal dates

Study protocol approved by Study Director: 05-Dec-2001
Study commenced: 22-Nov-2001 (Cytotoxicity assay treatment)
Study completed: 14-Feb-2002 (End of scoring - Main assay)

Study Director

S. Cinelli, Biol.D.

Archiving

The original data arising from this study and a copy of the final report consigned will be stored in the archives of Research Toxicology Centre S.p.A. for a period of five years from the date of consignment of the report. At the completion of this period the Sponsor will be contacted for despatch or disposal of the material, or further archiving. An aliquot of the test item will be retained within the archives of the testing facility for a period of ten years after which it will be destroyed.

3. MATERIALS

3.1 Test item

A 60 g sample of Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2), labelled as Bis (ethanolammonium)-hexahydroxoplatinat-losung, (Lot No.: A016-02), was received from DEGUSSA-DMC-2 on 28-Jan-2002 and was stored within the Formulation Unit at RTC at room temperature. The test item, an orange liquid, was contained in an opaque plastic bottle.
On 13-Feb-2002, a 6 g sub-sample was transferred from the Formulation Unit to the Department of Genetic and Cellular Toxicology and stored at approximately 4°C.

Solutions of the test item, as received, were prepared immediately before use in sterile culture medium on a volume/volume basis. In the main assay the solution at the maximum concentration was filter sterilized through a 0.45 µm filter before use. All test item solutions were used within 50 minutes of the initial formulation. No assay of test item stability, nor its concentration and homogeneity in solvent were undertaken. All dose-levels in this report are expressed to three significant figures.

3.2 Control items

The solvent used in this study was culture medium (EMEM minimal – RTC Lot No.: 008/044 and 005/238).

Solutions of ethylmethanesulphonate (batch no.: 29C-0523 obtained from Sigma Chemical Co.) were prepared in ethanol (batch no.: 0E059300E obtained from Carlo Erba) and served as positive controls in the absence of S9 metabolism. Solutions of 7,12-dimethylbenz(a)anthracene (batch no.: 106F-0505 obtained from Sigma Chemical Co.) were prepared in DMSO (batch no.: 421649/1 13001 obtained from Fluka AG) and served as positive control in the presence of S9 metabolism.

3.3 S9 Tissue Homogenate

Two batches of S9 tissue homogenate were used in this study and had the following characteristics:

Assay	S9 Batch	Protein content (mg/ml)	Aminopyrine demethylase activity (µM/g liver/5 min, formaldehyde production)
Toxicity test	2002/3	38.6 ± 2.69	3.98 ± 0.06
Main Assay	2002/6	35.9 ± 1.59	3.49 ± 0.16

Each S9 tissue fraction was prepared from the livers of five young male Sprague-Dawley rats which had received prior treatment with phenobarbital and betanaphthoflavone to induce high levels of xenobiotic metabolising enzymes.

The efficacy of the S9 tissue fractions was previously checked in an Ames test and produced acceptable responses with the indirect mutagens 2-aminoanthracene and benzo(a)pyrene, using *S. typhimurium* tester strain TA100.

3.4 Chinese Hamster V79 cells

Chinese hamster V79 cells were obtained from Dr. J. Thacker, MRC Radiobiology Unit, Harwell, UK. This cell line, V79 4(H) can be traced back directly to the original V79 isolate prepared by Ford and Yerganian. The karyotype, generation time, plating efficiency and mutation rates (spontaneous and induced) have been checked in this laboratory. The cells are checked at regular intervals for the absence of mycoplasmal contamination.
Permanent stocks of the V79 cells are stored in liquid nitrogen, and subcultures are prepared from the frozen stocks for experimental use.

3.5 Culture media

The following culture media were used:

EMEM Complete

Minimal medium	900	ml
Foetal Calf Serum	100	ml

Minimal Medium

Eagle's Minimal Essential Medium (10X)	58.7	ml
L-glutamine (200 mM)	5.9	ml
Sodium bicarbonate (7.5%)	15.7	ml
Non-essential amino acids (100X)	5.9	ml
Streptomycin sulphate 50 mg/ml		
Penicillin G 50.000 IU/ml	1.2	ml
Sterile bidistilled water	500	ml

4. METHODS

The methods used were in compliance with the Study Protocol (Appendix II).

4.1 Cytotoxicity assay

A preliminary cytotoxicity test was undertaken in order to select appropriate dose levels for the mutation assays. In this test a wide range of dose-levels of the test item was used; cell cultures were treated using the same treatment conditions as the mutation assays, and the survival of the cells was subsequently determined.

Treatments were performed both in the absence and presence of S9 metabolism; a single culture was used at each test point and positive controls were not included. Following treatment, cell monolayers were washed with Phosphate Buffered Saline (PBS); EMEM complete was added to the flasks which were than returned to the incubator at 37°C in a 5% CO_2 atmosphere (100% nominal relative humidity). The following day the cultures were trypsinised, counted, diluted and plated. After incubation for six days the colonies were stained with Giemsa solution and counted.

4.2 Mutation assay

4.2.1 Treatment of cell cultures

A single experiment was performed including negative and positive controls, in the absence and presence of S9 metabolising system. Two additional experiments were performed. In the first one, the test item was not filter sterilized and all treated cultures were contaminated a few days after treatment. In the second one unacceptably low plating efficiency values were obtained. This was probably due to technical problems. These experiments were not considered valid and generated data are not presented in this report, but are retained in the study file and archived as indicated in the study protocol.

Duplicate cultures were prepared at each test point, with the exception of the positive controls which were prepared in a single culture.

On the day before the experiment, sufficient numbers of 75 cm. sq. flasks were inoculated with 2 million freshly trypsinised V79 cells from a common pool. The cells were allowed to attach overnight prior to treatment.

The treatment media were prepared as follows:

Without S9 metabolism - Negative control and test item cultures

Minimal medium	8.1	ml
Hepes buffer (200 mM)	0.9	ml
Control or test item solution	1.0	ml
	10.0	ml

Without S9 metabolism – Positive control cultures

Minimal medium	8.9	ml
Hepes buffer (200 mM)	1.0	ml
Positive control solution	0.1	ml
	10.0	ml

With S9 metabolism - Negative control and test item cultures

Minimal medium	4.0	ml
S9 mix	5.0	ml
Control or test item solution	1.0	ml
	10.0	ml

With S9 metabolism – Positive control cultures

Minimal medium	4.9	ml
S9 mix	5.0	ml
Positive control solution	0.1	ml
	10.0	ml

The cultures were incubated at 37°C for three hours. At the end of the incubation period, the treatment medium was removed and the cell monolayers were washed with Phosphate Buffered Saline (PBS). Fresh complete medium was added to the flasks which were than returned to the incubator at 37°C in a 5% CO_2 atmosphere (100% nominal relative humidity) to allow for expression of the mutant phenotype.

4.2.2 Determination of survival

The following day, the cultures were trypsinised and an aliquot was diluted and plated to estimate the viability of the cells. A number of cells were then replated in order to maintain the treated cell populations; the number of cells taken forward was adjusted according to the expected viability of the cultures, to give one million viable cells.

4.2.3 Subculturing

On day 4 and day 6, the cell populations were subcultured in order to maintain them in exponential growth. The number of cells taken forward was adjusted according to the expected viability, to give at least one million viable cells seeded in F75 flasks.

4.2.4 Determination of mutant frequency

On day 6 and day 8, each culture was trypsinised, resuspended in complete medium and counted by microscopy.
After dilution, an estimated 1 x 10^5 cells were plated in each of five 100 mm tissue culture petri dishes containing medium supplemented with 6-thioguanine (at 7.5 µg/ml). These plates were subsequently stained with Giemsa solutions and scored for the presence of mutants.
After dilution, an estimated 200 cells were plated in each of three 60 mm tissue culture petri dishes. These plates were used to estimate Plating Efficiency (P.E.).

5. RESULTS

5.1 Solubility test

The test item was found to be soluble in sterile culture medium (EMEM minimal) at a concentration of 50.0 mg/ml. Since 1 ml of the test item solution is used in the preparation of each treatment culture, a maximum concentration of 5000 µg/ml, as indicated in the study protocol, was used in the cytotoxicity assay.

5.2 Cytotoxicity test

The test item was assayed at a maximum concentration of 5000 µg/ml and eight lower dose-levels: 2500, 1250, 625, 313, 156, 78.1, 39.1 and 19.5 µg/ml. At the end of the treatment incubation period, precipitation was observed at the four higher concentrations tested
Results are presented in Tables 1 and 2.

In the absence of S9 metabolic activation, survival was reduced to below the limit of detection at concentrations between 5000 and 625 µg/ml. At 313, 156 and 78.1 µg/ml severe toxicity was observed reducing survival to between 1 and 12% of the concurrent control value, while at the remaining concentrations moderate toxicity was observed. In the presence of S9 metabolic activation moderate toxicity was observed at the highest dose-level reducing survival to 40% of the negative control value. Based on these results, the maximum dose-levels for the first mutation assay were selected as 78.1 µg/ml for treatment in the absence of S9 metabolism and 5000 µg/ml in the presence.

5.3 Mutation assays

5.3.1 Experimental design

A single assay for mutation to 6-thioguanine resistance was performed using dose-levels indicated in the following table:

Assay No.:	S9	Dose-level (µg/ml)
1	-	78.1, 39.1, 19.5, 9.77, 4.88 and 2.44
1	+	5000, 2500, 1250, 625 and 313

Dose-levels were selected to cover a range from the maximum to little or no toxicity.

5.3.2 Survival after treatment

The survival data are shown in Tables 4, 5.

In Main assay 1, following treatment in the absence of S9 metabolism, a dose-related decline in survival was observed reaching approximately 24% of the control value at the highest concentration (78.1 µg/ml). In the presence of S9 metabolism, moderate toxicity was observed at all dose-levels assayed and survival was reduced to 24% of the concurrent control value at the highest concentration (5000 µg/ml). It should be noted that a depression in plating efficiency counts on Day 6 and Day 8 was observed at higher concentrations plated for mutation frequency, both in the absence and presence of S9 metabolism. This indicates that these cultures had not recovered from treatment.

5.3.3 Mutation results

The results of the mutation assays are presented in Tables 6-9 and summarized in Table 10.

Dose-related and large increases in mutant numbers or mutant frequency were observed following treatment with the test item, in the absence and presence of S9 metabolism. These increases were greater than five-fold the spontaneous mutation frequency, in both replicate cultures when analysed separately, at both expression times and so can be considered as clear evidence of mutation induction.

The mutant frequencies in the negative control cultures fell within the normal range. Treatment with the positive control items gave marked responses in all experiments indicating the correct functioning of the test system.

5.4 Osmolality and pH

The pH values and osmolality of the post-treatment media were determined and results are presented in Table 3. The addition of the test item solution did not have any obvious effect on the osmolality or pH of the treatment medium.

6. ANALYSIS OF RESULTS

6.1 Statistical analysis

The results of these experiments were subjected to an Analysis of Variance in which the effect of replicate culture, expression time and dose-level in explaining the observed variation was examined. For each experiment, the individual mutation frequency values at each test point were transformed to induce homogeneous variance and normal distribution. The appropriate transformation was estimated using the procedure of Snee and Irr (1981), and was found to be $y = (x + a)^b$ where $a = 0$ and $b = 0.275$. A three way analysis of variance was performed (without interaction) fitting to three factors:

1) Replicate culture : to identify differences between the replicate cultures treated.

2) Expression time : to identify differences in response at the expression times used.

3) Dose-level : to identify dose-related increases (or decreases) in response, after allowing for the effects of replicate cultures and expression time.

The analysis was performed separately with the sets of data obtained in the absence and presence of S9 metabolism. The analysis was achieved using the GLIM package. The F values obtained for each fitted factor and the corresponding probability values are given in Table 11.

Expression time and replicate culture were not significant factors in explaining the observed variation in the data. Both in the absence and presence of S9 metabolism, dose-level had a statistically significant effect. Mutation frequencies were greater at higher concentrations, indicating a dose-response relationship.

6.2 Criteria for outcome of assay

For a test item to be considered mutagenic in this assay, it is required that:

(i) There is a five-fold (or more) increase in mutation frequency compared with the solvent controls, over two consecutive doses of the test item. If only the highest practicable dose-level (or the highest dose-level not to cause unacceptable toxicity) gives such an increase, then a single treatment-level will suffice.

(ii) There must be evidence for a dose-relation (i.e. statistically significant effect in the ANOVA analysis).

The "five-fold increase" in mutant frequency above the concurrent negative control is used as arbitrary criteria for positive response and it was established in our laboratory based on analysis of variation of negative control data.
If spontaneous mutation frequency is in the upper part of the historical range, significance of mutation increase is evaluated case by case.

Historical control data are used to demonstrate biological relevance of the results obtained.

6.3 Evaluation

Large increases in mutant frequency were observed both in the absence and presence of metabolic activation. Analysis of variance shows a statistically significant effect of the test item. It is concluded that Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2) induces gene mutation in Chinese hamster V79 cells after *in vitro* treatment.

7. CONCLUSIONS

It is concluded that Dihydrogen hexahydroxyplatinate, compound with 2-aminoethanol (1:2) induces mutation in Chinese hamster V79 cells after *in vitro* treatment, in the absence and presence of S9 metabolic activation, under the reported experimental conditions.

8. TABLES 1 - 11

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DIHYDROGEN HEXAHYDROXYPLATINATE, COMPOUND WITH 2-AMINOETHANOL (1:2): GENE MUTATION IN CHINESE HAMSTER V79 CELLS

TABLE 1 - WITHOUT METABOLIC ACTIVATION

STUDY NO.: 9397

SOLVENT: Culture medium

EXPERIMENT NO.: Toxicity Test

Treatment	Dose-level µg/ml	Plate counts			Mean	Percentage survival
Solvent Control	0.00	169	167	129	155	100
Solvent Control	0.00	169	167	129	155	100
Test Item	19.5	97	85	93	92	59
Test Item	39.1	39	51	57	49	32
Test Item	78.1	23	20	14	19	12
Test Item	156	9	6	6	7	5
Test Item	313	0	3	1	1	1
Test Item	625	0	0	2	1	0
Test Item	1250	0	0	0	0	0
Test Item	2500	0	0	0	0	0
Test Item	5000	1	0	0	0	0

Absolute plating efficiency of negative control = 78%

DIHYDROGEN HEXAHYDROXYPLATINATE, COMPOUND WITH 2-AMINOETHANOL (1:2): GENE MUTATION IN CHINESE HAMSTER V79 CELLS

TABLE 2 - WITH METABOLIC ACTIVATION

STUDY NO.: 9397

SOLVENT: Culture medium

EXPERIMENT NO.: Toxicity Test

Treatment	Dose-level µg/ml	Plate counts			Mean	Percentage survival
Solvent Control	0.00	195	168	180	181	100
Solvent Control	0.00	195	168	100	154	85
Test Item	19.5	147	136	158	147	81
Test Item	39.1	119	124	148	130	72
Test Item	78.1	177	203	213	198	109
Test Item	156	157	161	152	157	87
Test Item	313	194	197	210	200	111
Test Item	625	141	131	135	136	75
Test Item	1250	135	136	146	139	77
Test Item	2500	102	120	122	115	63
Test Item	5000	64	68	83	72	40

Absolute plating efficiency of negative control = 91%

DIHYDROGEN HEXAHYDROXYPLATINATE, COMPOUND WITH 2-AMINOETHANOL (1:2):
GENE MUTATION IN CHINESE HAMSTER V79 CELLS

TABLE 3 - pH AND OSMOLALITY OF TREATMENT MEDIUM

STUDY NO.: 9397

SOLVENT: DMSO

Dose-level (µg/ml)	WITHOUT S9			Dose-level (µg/ml)	WITH S9		
		pH	mOsm/kg			pH	mOsm/kg
0.00	A	7.59	285	0.00	A	7.17	344
	B	7.57	285		B	7.17	346
2.44	A	7.54	288	313	A	7.16	345
	B	7.56	285		B	7.20	343
4.88	A	7.57	285	625	A	7.18	344
	B	7.56	286		B	7.14	346
9.77	A	7.57	287	1250	A	7.15	342
	B	7.56	286		B	7.16	343
19.5	A	7.56	285	2500	A	7.17	343
	B	7.56	285		B	7.17	343
39.1	A	7.57	285	5000	A	7.18	345
	B	7.62	287		B	7.19	345
78.1	A	7.57	285				
	B	7.57	285				

TABLE 4 - SURVIVAL AFTER TREATMENT WITHOUT METABOLIC ACTIVATION

STUDY NO.: 9397

SOLVENT: Culture medium

POSITIVE CONTROL: Ethylmethanesulphonate

SOLVENT: DMSO

EXPERIMENT NO.: 1

	Dose-level µg/ml		Plate counts			Mean	Percentage survival
	0.00	A	138	180	167		
		B	176	170	200	172	100
	2.44	A	180	199	186		
		B	186	178	188	186	108
	4.88	A	200	220	212		
		B	148	220	220	203	118
	9.77	A	126	140	133		
		B	199	180	180	160	93
	19.5	A	110	126	116		
		B	128	152	129	127	74
	39.1	A	110	108	107		
		B	113	105	124	111	65
	78.1	A	43	39	40		
		B	41	40	48	42	24
EMS	10.0		120	116	106	114	66

Absolute plating efficiency of negative control = 86%

DIHYDROGEN HEXAHYDROXYPLATINATE, COMPOUND WITH 2-AMINOETHANOL (1:2): GENE MUTATION IN CHINESE HAMSTER V79 CELLS

TABLE 5 - SURVIVAL AFTER TREATMENT WITH METABOLIC ACTIVATION

STUDY NO.: 9397

SOLVENT: Culture medium

POSITIVE CONTROL: 7,12-Dimethylbenz(a)anthracene

SOLVENT: DMSO

EXPERIMENT NO.: 1

Dose-level µg/ml		Plate counts			Mean	Percentage survival
0.00	A	153	154	147		
	B	148	152	173	155	100
313	A	79	78	77		
	B	115	93	111	92	60
625	A	98	108	111		
	B	97	83	85	97	63
1250	A	94	81	97		
	B	97	110	86	94	61
2500	A	86	90	93		
	B	80	64	80	82	53
5000	A	46	55	47		
	B	18	32	26	37	24
DMBA 10.0		54	46	53	51	33

Absolute plating efficiency of negative control = 77%

DIHYDROGEN HEXAHYDROXYPLATINATE, COMPOUND WITH 2-AMINOETHANOL (1:2): GENE MUTATION IN CHINESE HAMSTER V79 CELLS

STUDY NO.: 9397 TABLE 6 - MUTATION ASSAY - WITHOUT METABOLIC ACTIVATION

EXPERIMENT NO.: 1 EXPRESSION TIME: 6 days SOLVENT: EMEM minimal

POSITIVE CONTROL: Ethylmethanesulphonate SOLVENT: Ethanol

Dose-level µg/ml		Viability plate counts			P.E.	Mutation plate counts					Tot.	Mean	SD	MF	MF Pooled cultures
0.00	A	145	136	138	70	0	1	1	0	0	2	0.4	0.5	5.73	
	B	145	120	134	67	0	1	1	0	1	3	0.6	0.5	9.02	7.33
2.44	A	139	136	144	70	1	0	0	0	0	1	0.2	0.4	2.86	
	B	134	132	117	64	0	2	0	1	1	4	0.8	0.8	12.53	7.48
4.88	A	136	126	138	67	2	0	0	0	2	4	0.8	1.1	12.00	
	B	132	120	136	65	2	0	1	0	0	3	0.6	0.9	9.28	10.66
9.77	A	120	114	110	57	1	1	0	2	2	6	1.2	0.8	20.93	
	B	146	140	150	73	0	0	0	0	0	0	0.0	0.0	2.75 *	9.23
19.5	A	82	80	83	41	1	2	2	3	4	12	2.4	1.1	58.78	
	B	100	104	100	51	2	0	2	1	0	5	1.0	1.0	19.74	37.16
39.1	A	98	92	94	47	9	2	7	2	4	24	4.8	3.1	101.41	
	B	121	129	122	62	2	6	5	7	7	27	5.4	2.1	87.10	93.29
78.1	A	65	64	52	30	5	4	5	4	2	20	4.0	1.2	132.60	
	B	60	64	73	33	4	5	5	3	3	20	4.0	1.0	121.83	126.98
EMS 10.0		148	146	149	73	80	92	90	113	114	489	97.8	15.0	1324.60	

P.E. = Absolute plating efficiency
Tot. = Total number of mutant colonies
Mean = Mean of mutation plate counts
SD = Standard deviation of mutation plate counts
MF = Are mutation frequencies per million surviving cells
* = Mutation frequency, assuming 1 colony on mutation plates

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DIHYDROGEN HEXAHYDROXYPLATINATE, COMPOUND WITH 2-AMINOETHANOL (1:2): GENE MUTATION IN CHINESE HAMSTER V79 CELLS

STUDY NO.: 9397 TABLE 7 - MUTATION ASSAY - WITH METABOLIC ACTIVATION

EXPERIMENT NO.: 1 EXPRESSION TIME: 6 days SOLVENT: EMEM minimal

POSITIVE CONTROL: 7,12-Dimethylbenz(a)anthracene SOLVENT: DMSO

Dose-level µg/ml		Viability plate counts			P.E.	Mutation plate counts					Tot.	Mean	SD	MF	MF Pooled cultures
0.00	A	160	156	170	81	0	1	3	0	0	4	0.8	1.3	9.88	
	B	144	159	169	79	1	0	0	0	0	1	0.2	0.4	2.54	6.26
313	A	130	130	133	66	1	1	0	0	0	2	0.4	0.5	6.11	
	B	132	126	124	64	4	2	4	1	2	13	2.6	1.3	40.84	23.23
625	A	110	114	125	58	1	3	3	1	6	14	2.8	2.0	48.14	
	B	100	109	101	52	2	2	4	2	3	13	2.6	0.9	50.32	49.17
1250	A	111	115	121	58	5	3	7	5	6	26	5.2	1.5	89.91	
	B	98	108	111	53	8	5	3	4	3	23	4.6	2.1	87.07	88.55
2500	A	68	70	63	34	2	6	4	5	3	20	4.0	1.6	119.40	
	B	59	64	64	31	4	3	6	4	4	21	4.2	1.1	134.76	126.80
5000	A	70	82	88	40	5	8	6	5	10	34	6.8	2.2	170.00	
	B	63	54	50	28	6	7	7	4	9	33	6.6	1.8	237.13	197.54
DMBA 10.0		97	100	97	49	53	48	40	42	52	235	47.0	5.8	959.18	

P.E. = Absolute plating efficiency
Tot. = Total number of mutant colonies
Mean = Mean of mutation plate counts
SD = Standard deviation of mutation plate counts
MF = Are mutation frequencies per million surviving cells
* = Mutation frequency, assuming 1 colony on mutation plates

DIHYDROGEN HEXAHYDROXYPLATINATE, COMPOUND WITH 2-AMINOETHANOL (1:2): GENE MUTATION IN CHINESE HAMSTER V79 CELLS

STUDY NO.: 9397 TABLE 8 - MUTATION ASSAY - WITHOUT METABOLIC ACTIVATION

EXPERIMENT NO.: 1 EXPRESSION TIME: 8 days SOLVENT: EMEM minimal

POSITIVE CONTROL: Ethylmethanesulphonate SOLVENT: Ethanol

Dose-level µg/ml		Viability plate counts			P.E.	Mutation plate counts					Tot.	Mean	SD	MF	MF Pooled cultures
0.00	A	128	140	150	70	1	1	1	1	1	5	1.0	0.0	14.35	
	B	100	104	102	51	2	0	0	0	2	4	0.8	1.1	15.69	14.92
2.44	A	150	151	165	78	0	0	0	0	0	0	0.0	0.0	2.58 *	
	B	160	170	171	84	0	0	0	0	0	0	0.0	0.0	2.40 *	1.24 *
4.88	A	156	154	145	76	0	2	0	0	2	4	0.8	1.1	10.55	
	B	131	146	124	67	0	1	0	0	1	2	0.4	0.5	5.99	8.41
9.77	A	104	112	122	56	1	1	1	2	1	6	1.2	0.4	21.30	
	B	147	148	146	74	0	1	2	0	0	3	0.6	0.9	8.16	13.86
19.5	A	119	116	113	58	4	5	2	6	3	20	4.0	1.6	68.97	
	B	117	111	113	57	2	1	2	2	2	9	1.8	0.4	31.67	50.51
39.1	A	145	146	150	74	4	2	6	3	6	21	4.2	1.8	57.14	
	B	138	140	132	68	6	7	4	6	4	27	5.4	1.3	79.02	67.69
78.1	A	66	60	72	33	9	8	15	8	6	46	9.2	3.4	278.79	
	B	81	82	100	44	4	7	5	3	3	22	4.4	1.7	100.38	177.01
EMS 10.0		159	177	174	85	97	87	112	88	97	481	96.2	10.0	1131.76	

P.E. = Absolute plating efficiency
Tot. = Total number of mutant colonies
Mean = Mean of mutation plate counts
SD = Standard deviation of mutation plate counts
MF = Are mutation frequencies per million surviving cells
* = Mutation frequency, assuming 1 colony on mutation plates

DIHYDROGEN HEXAHYDROXYPLATINATE, COMPOUND WITH 2-AMINOETHANOL (1:2): GENE MUTATION IN CHINESE HAMSTER V79 CELLS

STUDY NO.: 9397 TABLE 9 - MUTATION ASSAY - WITH METABOLIC ACTIVATION

EXPERIMENT NO.: 1 EXPRESSION TIME: 8 days SOLVENT: EMEM minimal

POSITIVE CONTROL: 7,12-Dimethylbenz(a)anthracene SOLVENT: DMSO

Dose-level µg/ml		Viability plate counts			P.E.	Mutation plate counts					Tot.	Mean	SD	MF	MF Pooled cultures
0.00	A	156	138	159	76	3	2	1	1	2	9	1.8	0.8	23.84	
	B	137	125	139	67	0	0	0	0	0	0	0.0	0.0	2.99 *	12.65
313	A	119	110	120	58	0	2	2	0	1	5	1.0	1.0	17.19	
	B	140	150	150	73	1	2	1	4	1	9	1.8	1.3	24.55	21.29
625	A	107	91	96	49	4	4	2	6	3	19	3.8	1.5	77.55	
	B	129	90	123	57	6	1	4	1	1	13	2.6	2.3	45.61	60.38
1250	A	80	76	63	37	6	5	7	7	5	30	6.0	1.0	164.38	
	B	61	59	78	33	5	5	2	6	3	21	4.2	1.6	127.27	146.76
2500	A	69	60	60	32	6	4	10	9	20	49	9.8	6.2	311.11	
	B	44	50	59	26	6	5	4	6	5	26	5.2	0.8	203.92	263.16
5000	A	71	78	64	36	9	10	10	9	11	49	9.8	0.8	276.06	
	B	70	66	70	34	11	9	10	7	12	49	9.8	1.9	285.44	280.67
DMBA 10.0		58	48	65	28	45	52	39	50	52	238	47.6	5.6	1670.18	

P.E. = Absolute plating efficiency
Tot. = Total number of mutant colonies
Mean = Mean of mutation plate counts
SD = Standard deviation of mutation plate counts
MF = Are mutation frequencies per million surviving cells
* = Mutation frequency, assuming 1 colony on mutation plates

DIHYDROGEN HEXAHYDROXYPLATINATE, COMPOUND WITH 2-AMINOETHANOL (1:2): GENE MUTATION IN CHINESE HAMSTER V79 CELLS

TABLE 10 - SUMMARY TABLE

STUDY NO.: 9397

SOLVENT: EMEM minimal

EXPERIMENT NO.: 1

Without metabolic activation				With metabolic activation			
Dose-level µg/ml	%RS	MF day 6	MF day 9	Dose-level µg/ml	%RS	MF day 6	MF day 9
0.00	100	7.33	14.92	0.00	100	6.26	12.65
2.44	108	7.48	1.24*	313	60	23.23	21.29
4.88	118	10.66	8.41	625	63	49.17	60.38
9.77	93	9.23	13.86	1250	61	88.55	146.76
19.5	74	37.16	50.51	2500	53	126.80	263.16
39.1	65	93.29	67.69	5000	24	197.54	280.67
78.1	24	126.98	177.01				
EMS 10.0	66	1324.60	1131.76	DMBA 10.0	33	959.18	1670.18

%RS = Percentage relative survival
MF = Are mutation frequencies per million surviving cells
* = Mutation frequency, assuming 1 colony on mutation plates

DIHYDROGEN HEXAHYDROXYPLATINATE, COMPOUND WITH 2-AMINOETHANOL (1:2): GENE MUTATION IN CHINESE HAMSTER V79 CELLS

TABLE 11 - ANALYSIS OF VARIANCE

STUDY NO.: 9397

SOLVENT: EMEM minimal

Dimension	F. value	(d.f.)	P
In absence of S9 metabolism			
Expression time	0.09709	(1,24)	NS
Replicate culture	2.43050	(1,24)	NS
Dose-level	98.03975	(1,24)	P<0.001
In presence of S9 metabolism			
Expression time	2.09621	(1,28)	NS
Replicate culture	0..09621	(1,28)	NS
Dose-level	45.11470	(1,28)	P<0.001

9. APPENDIX I - Historical Control Data

HISTORICAL DATA OF NEGATIVE/SOLVENT CONTROL
(mutation frequencies per million surviving cells)

WITHOUT METABOLIC ACTIVATION		WITH METABOLIC ACTIVATION	
Day 6	**Day 8 / Day 9**	**Day 6**	**Day 8 / Day 9**
	Mean value		
8.80	10.6	9.10	11.8
	Standard deviation (σ_{n-1})		
7.56	8.15	8.16	10.2
	Number of experiments		
62	62	62	62
	Upper confidence limit		
28.3	31.6	30.2	38.1
	Observed range		
1.01 – 39.3	2.22 – 43.3	2.25 – 47.7	2.22 – 56.1

HISTORICAL DATA OF POSITIVE CONTROL
(mutation frequencies per million surviving cells)

WITHOUT METABOLIC ACTIVATION		WITH METABOLIC ACTIVATION	
Day 6	**Day 8 / Day 9**	**Day 6**	**Day 8 / Day 9**
	Mean value		
987	1001	654	667
	Standard deviation (σ_{n-1})		
257	292	165	205
	Number of experiments		
62	62	62	62
	Lower confidence limit		
323	248	229	140
	Upper confidence limit		
1650	1753	1080	1195
	Observed range		
606 – 2427	391 – 2631	253 – 1100	214 – 1173